Exhibit A

Caspase and Proteasome Activity during Staurosporin-Induced Apoptosis in Lens Epithelial Cells

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Purpose. To determine what caspases are activated during staurosporin-induced apoptosis in cultured bovine lens epithelial cells (BLECs), to study the time course of caspase activation in relation to morphologic changes, and to investigate the effect of caspase and/or proteasome inhibition on apoptosis.

Methods. BLECs were incubated with staurosporin at different concentrations or for different times. Phosphatidylserine (PS) externalization was detected by annexin-V labeling, nuclear morphology was studied by staining with Hoechst 33342 stain (Hoechst, Frankfurt, Germany), and the percentage of apoptotic cells was determined by the TdT-dUTP terminal nick-end labeling (TUNEL) assay. The activity of caspase-1, -2, -3, -4, -8, and -9 as well as the chymotrypsin-like activity of the proteasome was measured by the use of fluorogenic peptide substrates. Inhibition of the proteasome was performed by incubation with 10 μM lactacystin, and caspases were inhibited by 1 μM Z-DEVD-FMK or 20 μM Z-VAD-FMK.

Results. Staurosporin treatment caused a dose- and time-dependent increase in the number of apoptotic cells and in caspase-3 activity. Activation of caspase-2, -4, -8, and -9 was also seen. Caspase activity was increased after 3 hours' incubation with 1 μM staurosporin, which is also the time when most cells became annexin-V–positive. Nuclear changes indicative of apoptosis, viewed with both Hoechst and TUNEL staining, appeared after 4 to 6 hours of staurosporin incubation. Incubation of BLECs with lactacystin caused reduction of proteasome activity and increased apoptosis, evidenced in both the TUNEL assay and caspase-3 activation. Preincubation of lens epithelial cells with caspase inhibitors caused complete inhibition of lactacystin- or staurosporin-induced caspase-3 activation (Z-DEVD-FMK/Z-VAD-FMK) and also of caspase-2, -4, -8, and -9 (Z-VAD-FMK), but the reduction in TUNEL-positive cells was only partial. PS translocation and DNA fragmentation after staurosporin treatment occurred despite complete caspase blockade.

Conclusions. Staurosporin-induced apoptosis in BLECs involves activation of several caspases. Inhibition of the proteasome causes caspase-3 activation and apoptosis. Both staurosporin- and lactacystin-induced apoptosis can be executed in a caspase-independent manner. The present data are useful for understanding of proteolytic mechanisms during apoptosis in lens epithelial cells, which may be an important event in normal lens development as well as in some types of cataract. (*Invest Ophthalmol Vis Sci.* 2000;41:2623–2632)

Apoptosis, or programmed cell death, occurs as a physiological phenomenon during normal embryonal development and is involved in cell turnover throughout life. It can also be induced by external influence such as radiation, hormones, or viral infection and has been implied in several diseases.

The lens epithelium is a single layer of cuboidal cells at the anterior surface of the lens with mitosis confined to the periphery. As the cells approach the equator, they start to differentiate, elongate, and eventually form lens fibers.[1] During this process, cell nuclei and all organelles are lost. Apoptosis has been demonstrated in lens epithelial cells during the earliest stages of this differentiation[2] and a member of the caspase family (caspase-3–like) has been reported to be activated during rodent lens cell differentiation.[3] Most likely, interference with the apoptotic process plays a role in congenital cataract or in adult cataract such as posterior subcapsular cataract, for which disturbed differentiation has been suggested as a cause.[4] Data have also been presented indicating involvement of apoptosis in age-related cataract in general and in posterior capsular opacification.[5,6]

Caspases are a family of proteases thought to be the most important effector molecules that induce apoptosis. They were

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first discovered when it was observed that the gene product of *ced-3*, which is essential for programmed cell death during development of the nematode *Caenorhabditis elegans*, is highly similar to mammalian interleukin-1β-converting enzyme (ICE or caspase-1).[7,8] Since then, several caspases have been described; to date, 13 mammalian caspases are known, but their different roles and targets in apoptosis are not yet fully understood. They share several similarities in structure and substrate specificity. The name caspase refers to their being cysteine proteases with an unusual and absolute requirement of an aspartic acid residue at the P_1 position (for reviews see References 9 and 10).

Caspases are synthesized constitutively as inactive proenzymes, a common feature of proteases, and are activated either autocatalytically or by other proteases. A catalytic cascade, much resembling the complement or clotting cascade have been suggested for caspase activation.[11] This cascade can be initiated by several factors. Data indicate that the route for caspase activation differs depending on the proapoptotic stimuli and that not all caspases are active in all mechanisms. Apoptosis, through so-called death receptors, involves activation of caspase-8, whereas cytotoxic agents activate the cascade through caspase-9.[12,13] Recent data also provide evidence of caspase-independent induction of apoptosis.[14] In this study, activation of several caspases was detected in bovine lens epithelial cells (BLECs) during staurosporin-induced apoptosis, a commonly used way of provoking programmed cell death. Caspase activation coincided with or preceded morphologic changes typical of apoptosis.

Proteases other than the caspases have been implicated in apoptosis. Calpains have been suggested to have a role in apoptosis-related fodrinolysis,[15] and the proteasome is known to degrade small short-lived regulatory proteins,[16] some of which could be important in regulation of the cell cycle. Data are conflicting on the involvement of these proteases in apoptosis and their presumed roles. This study investigated proteasome activity in parallel with caspase activation and also looked at the effects of proteasome inhibition.

The effect of caspase inhibition during lactacystin or staurosporin incubation was also investigated. Although all caspases investigated were completely inhibited by a pancaspase inhibitor, only partial reduction of the number of apoptotic cells was seen. It is the first time to our knowledge, that the activity of a large number of caspases has actually been measured after treatment with a caspase inhibitor, in parallel with quantification of apoptotic cells. Morphologic apoptotic events were also studied during staurosporin treatment, in the absence and presence of a pancaspase inhibitor.

Materials and Methods

Cell Culture

Bovine eyes (50 eyes for the entire study) were obtained from a local abattoir (SCAN West, Skara, Sweden) and lenses removed within 4 hours after death under aseptic conditions. The entire eye was dipped in 70% ethanol for 30 seconds before it was opened from the posterior. The dissection procedure was performed with sterile instruments under a laminar flow hood. After removal of the vitreous, the zonula fibers were cut, and the lens was lifted to a sterile filter paper where it was gently rolled to remove any adhering vitreous or ciliary body pigment. The lens was then pinned to a paraffin plate, the capsule epithelium cut along the equator, and the whole lens placed upside down in a 35-mm^2 culture dish (Techno Plastic Products, Trasadingen, Switzerland). The edges of the lens capsule were pressed down to the plastic surface by fine forceps and the bulk of the lens lifted from the dish, leaving the pieces of capsule epithelium adhering to the plastic, cell side up. Some drops of culture medium were applied to the epithelium specimens to prevent drying, and the dishes were put in a humidified CO_2 incubator (5% CO_2, 37°C) for approximately 2 hours to allow firm attachment of the capsules. Another 1 ml of culture medium was then added, and the capsules were left for 2 days before the first trypsinization. Typically, BLECs from 10 explants were pooled and subcultured in 25- or 80-cm^2 culture flasks (Nuncion; Naige Nunc, Napierville, IL) by trypsin-EDTA treatment (0.25%, from Sigma, St. Louis, MO). The medium, which was changed twice a week, was M199 with 10% fetal calf serum, supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (all from Sigma). For most experiments, cells were seeded in 96-well culture plates suitable for fluorescence assays (Corning Costar, Cambridge, MA) at a density of approximately 10^5 cells/well and left overnight to yield a confluent monolayer. For annexin labeling, cells were seeded on 13-mm round coverslips (BDH, Poole, UK) in 24-well culture dishes (TPP). Cells used in experiments were generally from the third to eight passage. Cell morphology did not change between those passages, and no increase or decrease of proteasome or caspase-3 activity in untreated cells was seen with passaging.

Incubation with Staurosporin, Lactacystin, and Caspase Inhibitors

Staurosporin from *Streptomyces* (Sigma) was kept in a stock solution of 10 mM in 100% dimethyl sulfoxide (DMSO) and diluted to a final concentration of 1 μM in serum-free medium before incubation. Controls were incubated with corresponding concentration of DMSO. When the effect of staurosporin concentration was investigated, final concentrations of 15.6, 31, 62.5, 125, 250, 500, and 1000 nM were used. Most incubation periods were 24 hours, but for investigation of the time course of staurosporin-induced apoptosis, incubation was stopped at 0, 1, 2, 3, 4, 6, 8, and 24 hours after addition of the agent.

A stock solution of 1 mM lactacystin (100% aqueous; Calbiochem, La Jolla, CA) was diluted in serum-free medium to a final concentration of 10 μM. The caspase-3 inhibitor Z-DEVD-FMK (Clontech, Palo Alto, CA) and the pancaspase inhibitor Z-VAD-FMK (Enzyme Systems Products, Livermore, CA) were prepared as stock solutions in 100% DMSO (1 and 20 mM, respectively). Final concentrations in serum-free medium were 1 μM for Z-DEVD-FMK and 20 μM for Z-VAD-FMK. Cells were incubated with lactacystin or caspase inhibitors 1 hour before addition of staurosporin. Control wells were incubated with corresponding DMSO concentrations.

For subsequent proteolytic assays, incubations were stopped by removing the medium and freezing the 96-well culture plates at −80°C, where they were stored until use (within 1 month). For TdT-dUTP terminal nick-end labeling (TUNEL) assays, medium was removed and cells fixed with 4% paraformaldehyde for 30 minutes at room temperature.

Annexin-V Assay

Annexin V is a small Ca^{2+}-dependent protein with high affinity for phosphatidylserine (PS).[17] In normal living cells, PS is located in the inner layer of the cell membrane only, but in apoptotic cells this phospholipid is translocated to the outer leaflet. PS exposure on the surface of cells functions as tags for specific recognition for phagocytosis by macrophages or neighboring cells.[18] Annexin V was used to detect apoptosis at an early stage in BLECs together with propidium iodide, which binds to DNA in cells that have lost membrane integrity (necrotic or late apoptotic cells). In accordance with manufacturer's instructions (Boehringer–Mannheim, Mannheim, Germany), 20 μl fluorescein-labeled annexin V and 20 μl propidium iodide (ready-to-use solutions) were diluted in 1 ml Hepes buffer. To enable visualization of all cell nuclei and to study nuclear morphology, 20 μl of the cell-permeable DNA-binding agent Hoechst 33342 (Hoechst, Frankfurt, Germany) was added to the annexin-V kit mixture. Hoechst 33342 was prepared as a 0.5-mg/ml stock solution in distilled water and was stable at 4°C for at least 6 months. Cells grown on coverslips were rinsed with phosphate-buffered saline (PBS; 0.02 M, pH 7.4) and incubated with the annexin-V solution for 10 minutes, after which the coverslips were mounted on microscope slides with glycerol and immediately viewed in a microscope (Optiphot 2; Nikon, Tokyo, Japan). Pictures were captured using a color-intensified 3CCD camera (model C5810; Hamamatsu, Hamamatsu City, Japan).

TUNEL Assay

TUNEL staining was performed according to the manufacturer's instructions (Boehringer–Mannheim). In short, fixed cells were rinsed three times with PBS and subsequently permeabilized with 0.1% Triton X 100 in 0.1% sodium citrate on ice for 10 minutes. After a rinse with PBS, cells were incubated with TUNEL reaction mixture (TdT and fluorescein-labeled nucleotides) for 60 minutes at 37°C in a humid chamber. For negative controls, TdT was excluded from the reaction mixture. For positive controls, permeabilized cells were preincubated with DNase I (80 U/ml in 0.15 M NaCl; Sigma) for 10 minutes at room temperature and then incubated with TdT-containing reaction mixture. After three rinsings with PBS, cells were incubated with anti-fluorescein antibody from sheep, conjugated with alkaline phosphatase, for 30 minutes at 37°C. Another washing with PBS was followed by a 30-minute incubation with fast red tablets dissolved in 0.1 M Tris-HCl (pH 8.2; Boehringer–Mannheim) at room temperature. Excess fast red reagent was removed by washing with PBS, and cells were then viewed in an inverted phase–contrast microscope (TMS-F; Nikon) to enable counting of unstained cells and TUNEL-positive cells. The percentage of TUNEL-positive cells in relation to the total number of cells was determined by counting at least 300 cells in three different fields. Mean ± SD was calculated from three separate culture wells, and the experiment was performed in triplicate two or three times.

Proteolytic Assays

Frozen lens epithelial cells were disrupted by addition of 100 μl of room tempered 3-([3-cholamidopropyl] dimethylammonio)-2-hydroxy-1-propanesulfonate (CHAPS)-containing buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA, 1 mM EGTA, 3 mM NaN_3, and 0.2% CHAPS [pH 7.3]) per well. The CHAPS buffer was supplemented with trypsin inhibitor (final concentration 5 μg/ml), pepstatin (0.5 μg/ml), leupeptin (1.25 μg/ml), and phenylmethylsulfonyl fluoride (0.5 mM) to minimize activity of proteases other than the desired ones. All protease inhibitors were from Sigma. Preincubation with inhibitor-containing CHAPS buffer was continued for at least 30 minutes in room temperature, after which 20 μl was removed for protein determination (see description later). In culture wells intended for measurement of proteasome activity, CHAPS buffer without inhibitors was used. For investigation of in vitro inhibition of proteasome or caspase-3 activity, lactacystin was included in the CHAPS buffer (10 μM final concentration in the assay).

The following synthetic substrates were used to measure caspase activity: caspase-1, Acetyl-Trp-Glu-His-Asp-7-amido-4-methylcoumarin (Ac-WEHD-AMC; Bachem, Bubendorf, Switzerland); caspase-2, Acetyl-Val-Asp-Val-Ala-Asp-7-amino-4-trifluoromethylcoumarin (Ac-VDVAD-AFC; Enzyme Systems

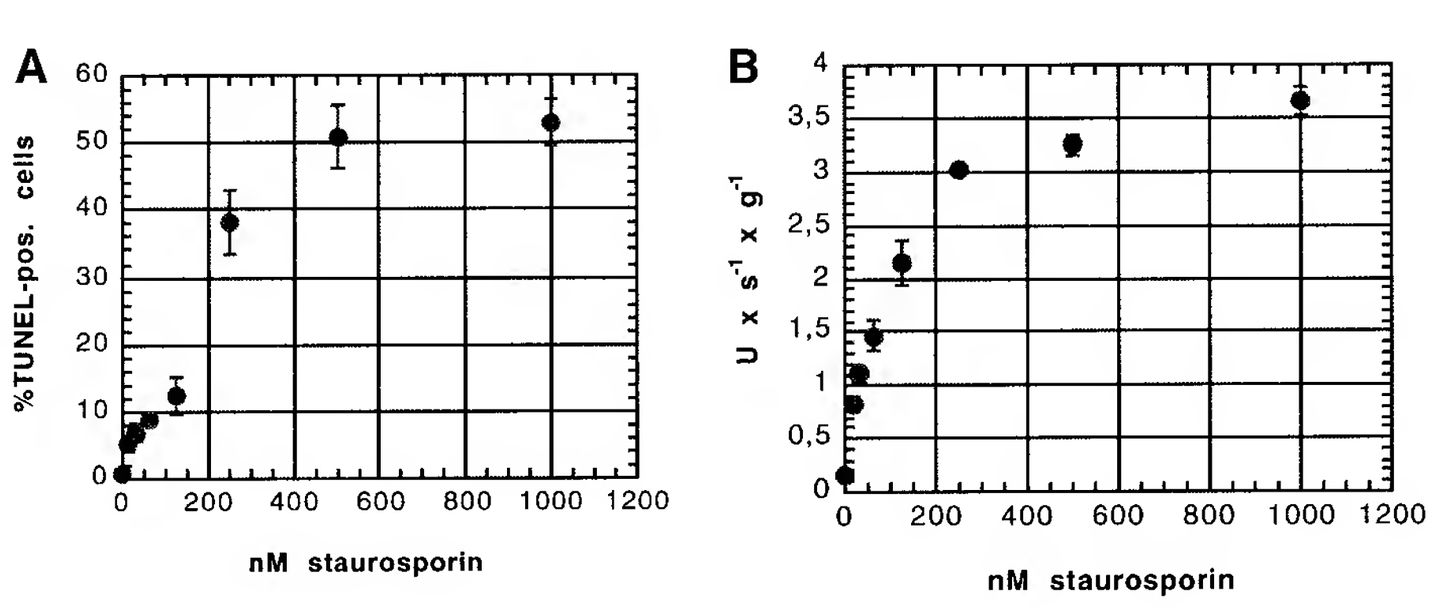

FIGURE 1. BLECs incubated with different concentrations of staurosporin for 24 hours exhibited an increased number of TUNEL-positive cells (**A**) and increased caspase-3 activity (**B**). The percentage of TUNEL-positive cells compared with the total number of cells was calculated by counting at least 300 cells per culture well. Caspase-3 activity was measured using Ac-DEVD-AMC as substrate. Increase of the fluorescent cleavage product was detected over time and activity expressed as relative fluorescence units per second and gram protein. Data presented are mean ± SD from three separate culture wells.

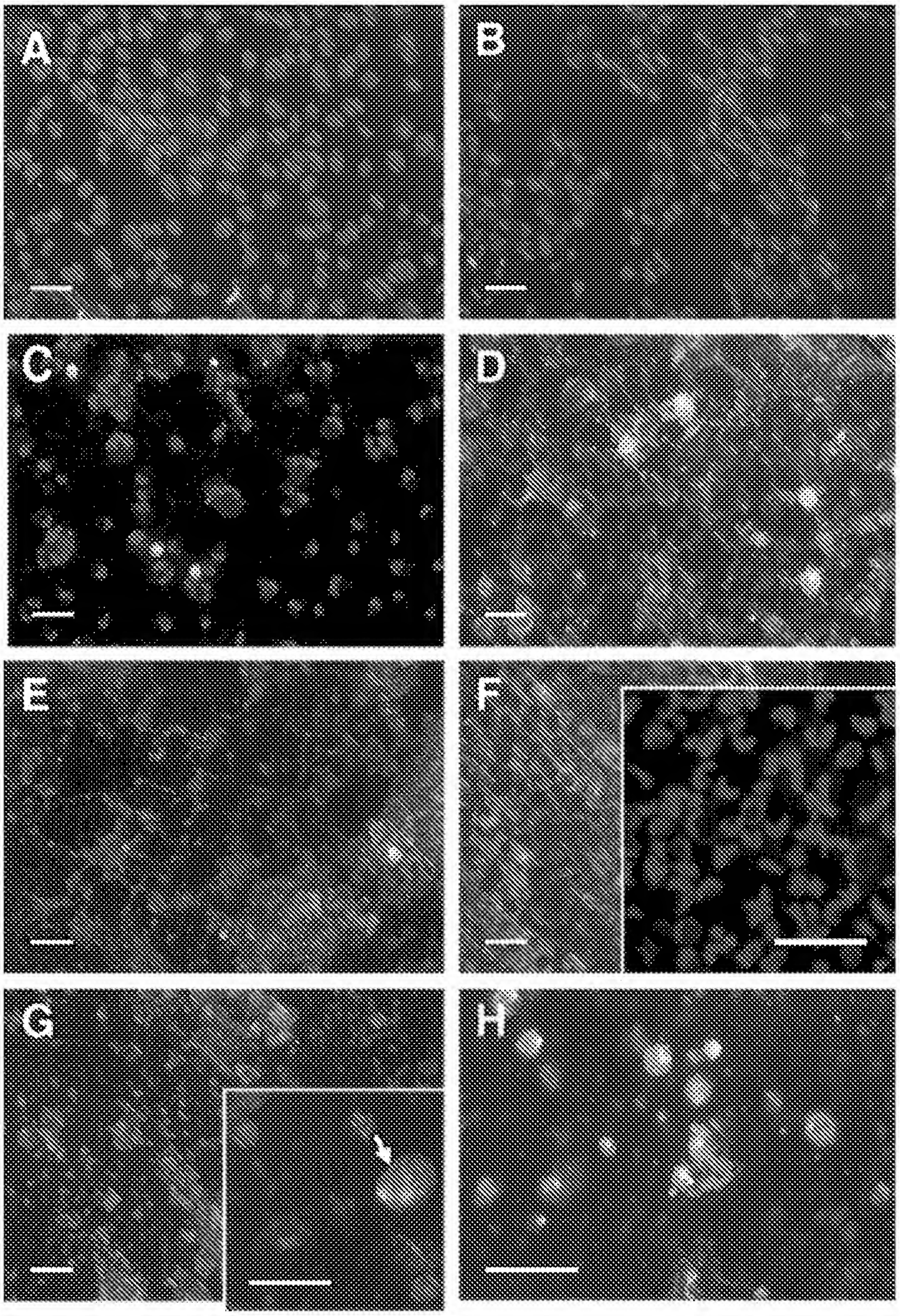

FIGURE 2. Time course of apoptosis in BLECs exposed to 1 μM staurosporin. Photographs were taken at 0 (**A**), 1 (**B**), 2 (**C**), 3 (**D**), 4 (**E**), 6 (**F**), 8 (**G**), and 24 (**H**) hours. At these time points, cells were freshly stained with Hoechst 33342 (*blue*), annexin V (*green*), and propidium iodide (*red*). Note cell shrinkage after staurosporin exposure compared with the cells at the start of the incubation (**A**). Annexin-labeling was apparent at 3 hours and later, indicating PS exposure to the outer leaflet of cell membranes, an early marker of apoptosis (**D** through **H**). Condensed and fragmented nuclei were seen at 6 hours and later (**F**, inset; Hoechst staining), and at these late stages, engulfment of apoptotic bodies could be seen (**G**, *inset*; *arrow*). Propidium iodide staining, indicative of loss of membrane integrity as in necrosis or late apoptosis, occurred in scattered cells at all time points (**A**, **C**, **D**, **F**, **G**, **H**). After 24 hours of incubation with 1 μM staurosporin, most cells showed uptake of propidium iodide (**H**). Scale bar, 50 μm.

Products); caspase-3, Acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC; Calbiochem); caspase-4, Acetyl-Leu-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (Ac-LEVD-AFC; Enzyme Systems Products); caspase-8, Acetyl-Ile-Glu-Thr-Asp-7-amido-4-methylcoumarin (Ac-IETD-AMC; Peptide Institute, Osaka, Japan); and caspase-9, Acetyl-Leu-Glu-His-Asp-7-amido-4-methylcoumarin (Ac-LEHD-AMC; Peptide Institute). All caspase substrates, except Ac-DEVD-AMC, were prepared as stock solutions of 10 mM in 100% DMSO. The Ac-DEVD-AMC substrate was dissolved in water as a 1-mM stock. Before the assay, all caspase substrates were diluted, yielding final concentrations of 28 μM, a concentration chosen on the basis of recent investigations about the K_m values for peptide sequences preferred by different caspases.[19] For measurement of the chymotrypsin-like peptidase activity of the proteasome, Succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Suc-LLVY-AMC; Bachem) was taken from a stock of 40 mM (100% DMSO) to yield a final concentration of 50 μM. All substrates were diluted in 50 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA, 1 mM EGTA, and 3 mM NaN_3 [pH 7.3], with the addition of 2 mM dithiothreitol (final concentration in the assay).

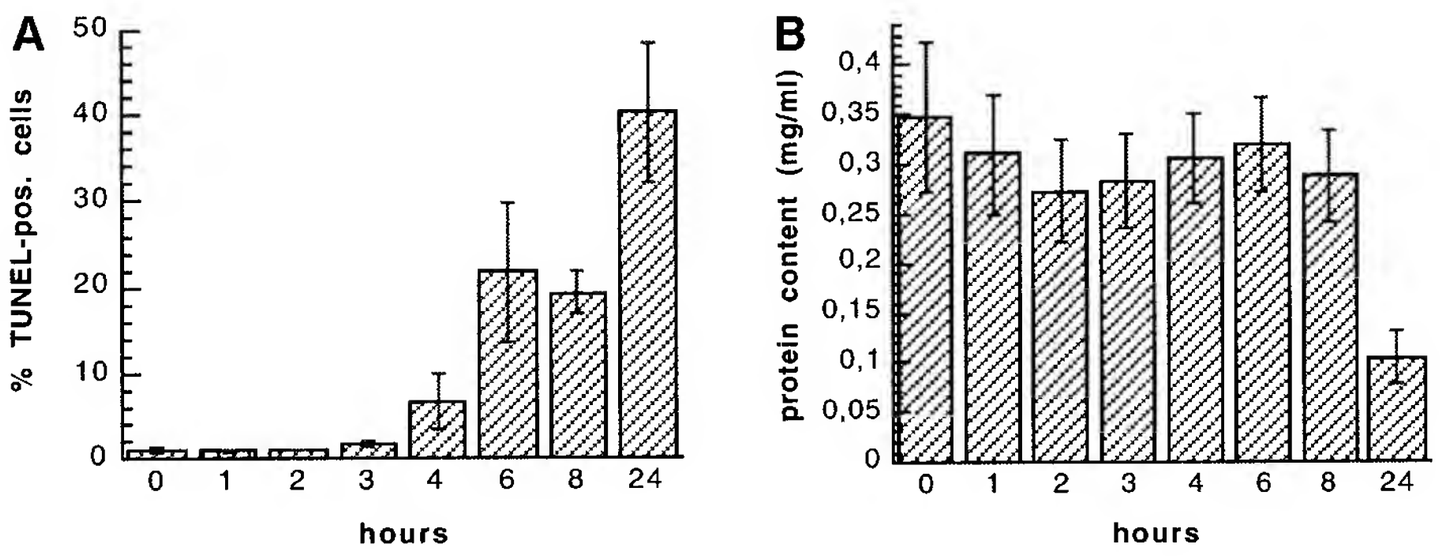

FIGURE 3. The number of TUNEL-positive BLECs in relation to the total number of cells (**A**) and the protein content in the culture wells (**B**) during 24 hours of incubation with 1 μM staurosporin. A small increase in the percentage of TUNEL-positive cells could be seen after 3 hours and was more prominent at 4 hours and later. At least 300 cells were counted in three different fields. Data are expressed as mean ± SD of three separate culture wells. The protein content was determined in cell lysates before proteolytic measurements. No difference was seen in the protein concentration during the first 8 hours, but after 24 hours, substantial protein loss occurred from the cultures.

To each culture well, each containing disrupted BLECs and 80 μl CHAPS buffer, 100 μl of substrate solution was added and fluorescence of the cleavage product measured over time at 37°C in a microplate spectrofluorometer (SpectraMax Gemini; Molecular Devices, Sunnyvale, CA). For AMC substrates, excitation wavelength was 380 nm, emission 440 nm; for AFC substrates, excitation was 400 nm and emission 505 nm. Activity was normally measured during 3 to 5 hours and V_{max} determined by computer (SOFTmax PRO ver. 2.6; Molecular Devices). Proteolytic activity was expressed as relative fluorescence units per second and gram of protein. In experiments in which time course of apoptosis was studied, activity is expressed as percentage of activity compared with the activity at $t = 0$ hours. Mean ± SDs from triplicate wells are shown from experiments performed on two or three separate occasions. For statistical analyses, two-tailed Student's *t*-tests for unmatched data were used. Only differences that were statistically significant in all experiments (and performed at least on three separate occasions) are denoted as such.

Protein Determination

Aliquots of 20 μl CHAPS buffer with BLEC lysates were taken for protein determination, using a BCA protein assay (Pierce, Rockford, IL) with bovine serum albumin as standard. Absorbance was measured at 570 nm in a microplate reader (Emax, software; SOFTmax ver. 2.01; Molecular Devices).

Results

Incubation with staurosporin caused a concentration-dependent increase both in the number of TUNEL-positive cells and in caspase-3 activity (Figs. 1A, 1B). After 24 hours of incubation with staurosporin, the percentage of TUNEL-positive cells showed a plateau at 500 nM staurosporin, and concentrations above 250 nM caused only a small increase in activity of caspase-3. Half-maximal response was obtained at 240 nM staurosporin (with respect to the percentage of TUNEL-positive cells) and 160 nM (caspase-3 activity). The dose–response curve for TUNEL-positive cells (Fig. 1A) also showed a component that required only 15 nM staurosporin for half-maximal response.

Annexin-V-labeling of staurosporin-treated cells was first evident after 3 hours of incubation and was more intense after 6 hours (Fig. 2A-H). Nuclear staining with Hoechst 33342 revealed morphologic changes indicative of apoptosis, such as chromatin condensing and nuclear fragmentation. Although sporadic apoptotic nuclei could be seen at earlier time points, they became frequent after 4 to 6 hours of staurosporin incubation (Fig. 2F, inset). Such small apoptotic nuclei could be seen in isolated apoptotic bodies and occasionally inside big cells, suggesting phagocytosis by neighboring cells (Fig. 2G, inset). Cells stained with propidium iodide, indicating necrosis, were scarcely seen during the first 8 hours of staurosporin incubation. After 24 hours, however, a majority of the cells had lost their membrane integrity and were stained by propidium iodide (Fig. 2H).

The frequency of TUNEL-positive cells at the start of incubation was approximately 1% of all cells (Fig. 3A). This number started to increase after 4 hours, and after 6 hours 22% of the cells were stained. After 24 hours of incubation, 40% of staurosporin-treated BLECs were TUNEL positive. The protein content at the end of the incubation period was approximately 30% of the initial protein content in the culture wells, indicating cell detachment (Fig. 3B). During the first 8 hours, however, the protein concentration was stable.

The activity of caspase-1, as measured by the Ac-WEHD-AMC substrate, did not change during staurosporin incubation (Fig. 4A). Caspase-2 and -3 showed substantial increases in activity after 3 hours and continued to increase during the incubation (Figs. 4B, 4C). The activity of caspase-3 was predominant compared with the other caspases, both in relative terms and in absolute measurements. Caspase-4 exhibited a slight elevation early during the staurosporin treatment, but increased more rapidly after 6 hours (Fig. 4D). Caspase-8 and -9 activity began to increase after 2 to 3 hours and continued to increase slowly (Figs. 4E, 4F). Proteasome activity was stable during the first 8 hours of staurosporin incubation and decreased after 24 hours (Fig. 5).

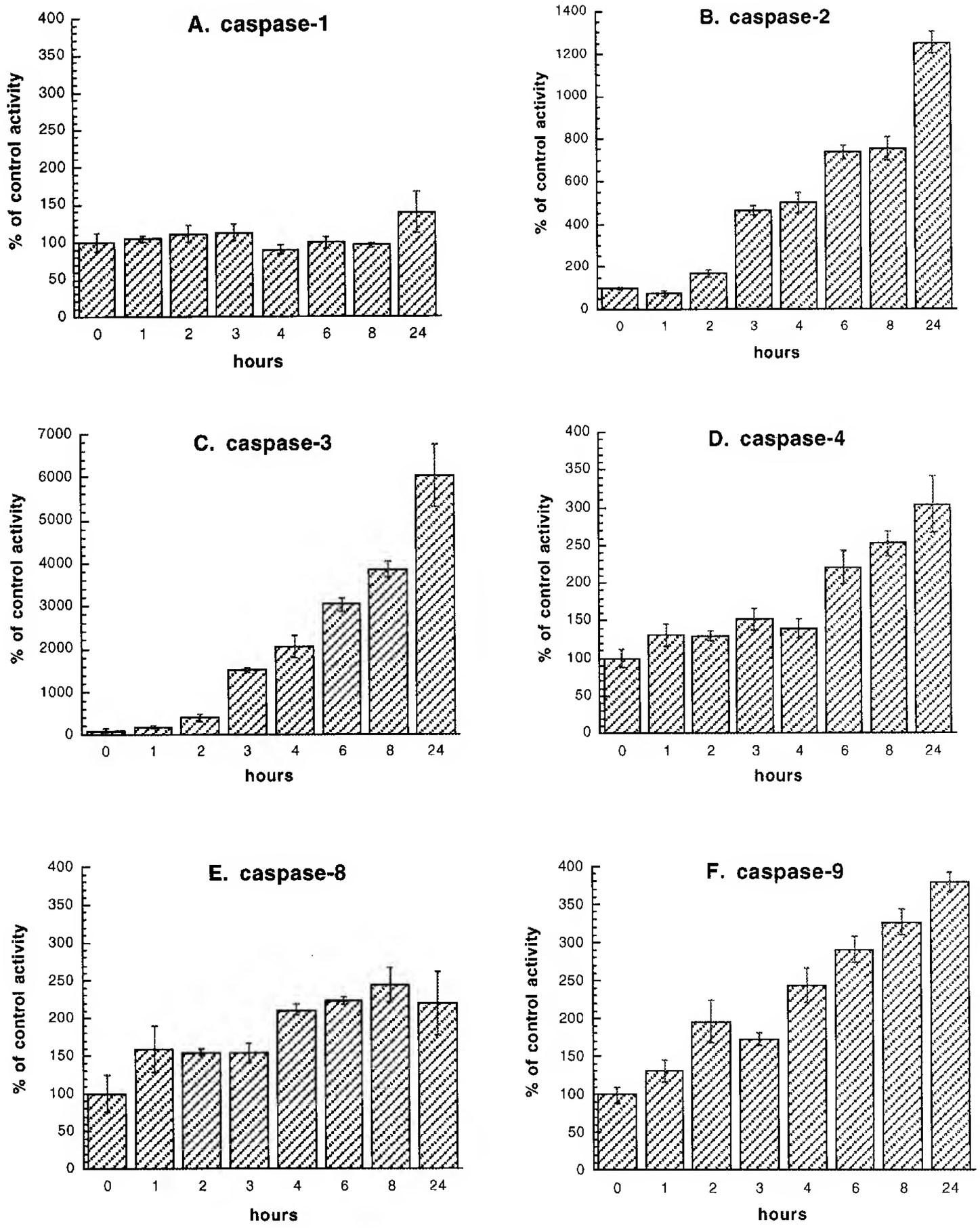

FIGURE 4. Activity of several caspases measured during incubation with 1 μM staurosporin for 24 hours. Fluorogenic substrates used were Ac-WEHD-AMC (**A**; caspase-1), Ac-VDVAD-AFC (**B**; caspase-2), Ac-DEVD-AMC (**C**; caspase-3), Ac-LEVD-AFC (**D**; caspase-4), Ac-IETD-AMC (**E**; caspase-8), and Ac-LEHD-AMC (**F**; caspase-9). Activity is expressed as percentage of activity at $t = 0$ hours (control activity). Means ± SDs of three separate culture wells are shown.

Incubation of BLECs with 10 μM lactacystin for 25 hours in culture caused an 86% reduction of proteasome activity when measured with Suc-LLVY-AMC (Table 1). This inhibitory effect on the proteasome was similar to that seen after lactacystin treatment in vitro. Treatment with lactacystin in culture also lead to apoptosis, revealed by an increased number of TUNEL-positive cells and an increase in caspase-3 activity. If cells were preincubated with lactacystin for 1 hour before addition of 1 μM staurosporin and then incubated for another 24 hours, there was no difference in the number of TUNEL-positive cells, with or without lactacystin. Caspase-3 activity was slightly higher if both lactacystin and staurosporin were present compared with staurosporin alone, but this difference was not significant. Proteasome activity decreased 59% after a 24-hour incubation with staurosporin in culture, but simultaneous incubation with lactacystin and staurosporin inhibited proteasome activity to the same extent as with lactacystin treatment only. Lactacystin in vitro had no significant effect on caspase-3 activity, neither on basal caspase-3 activity (no previous staurosporin incubation) nor after activation by staurosporin.

Preincubation of BLECs with the caspase-3 inhibitor Z-DEVD-FMK or the pancaspase inhibitor Z-VAD-FMK 1 hour before addition of 10 μM lactacystin or 1 μM staurosporin caused complete inhibition of Ac-DEVD-AMC hydrolytic activity (Table 2). The increased number of TUNEL-positive cells that was seen after lactacystin incubation was not blocked by caspase inhibitors, Z-DEVD-FMK or Z-VAD-FMK, but the staurosporin-induced increase in the percentage of TUNEL-positive cells was partially inhibited by Z-VAD-FMK. Activation of caspase-2, -4, -8, and -9 by staurosporin was also prevented by preincubation with 20 μM Z-VAD-FMK in culture (Table 3).

To investigate the effect of the pancaspase inhibitor Z-VAD-FMK on morphologic characteristics of apoptosis, such

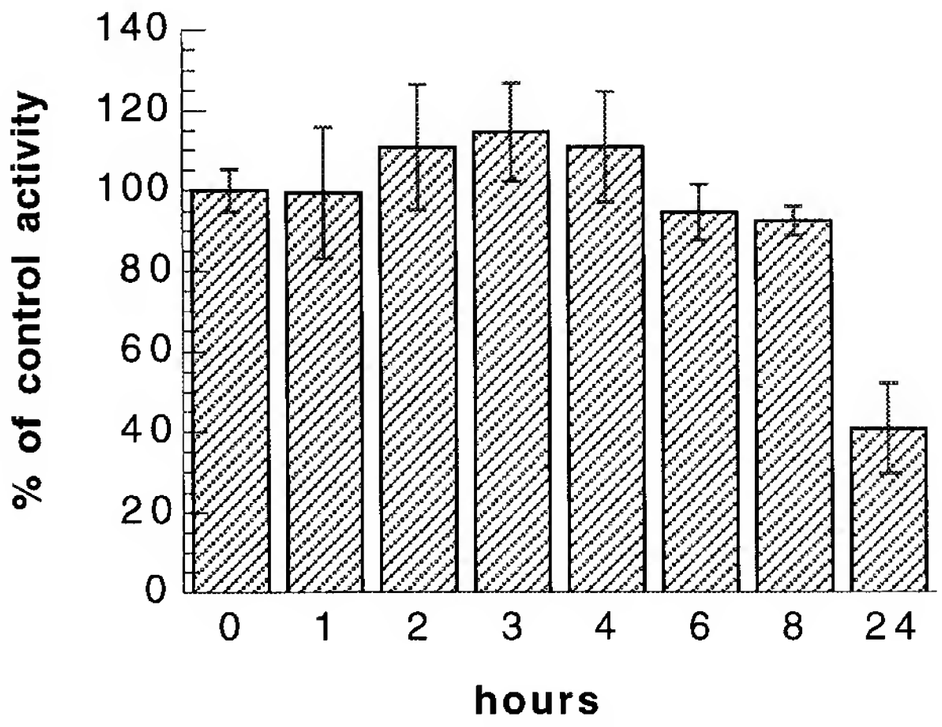

FIGURE 5. The chymotrypsin-like peptidase activity of the proteasome as measured by Suc-LLVY-AMC hydrolysis. BLECs were incubated with 1 μM staurosporin for 1, 2, 3, 4, 6, 8, and 24 hours. Activity is expressed as a percentage of activity before staurosporin treatment. Means ± SDs of three separate culture wells are shown.

as PS translocation and nuclear condensation and fragmentation, cells were pretreated with 20 μM Z-VAD-FMK for 1 hour before addition of 1 μM staurosporin. After 3 or 6 hours of staurosporin exposure, cells were freshly stained with annexin-V, Hoechst 33342, and propidium iodide. PS exposure and nuclear fragmentation was evident both in the absence and presence of Z-VAD-FMK.

DISCUSSION

Cell Morphology and Caspase Activation after Staurosporin Treatment

Staurosporin, a metabolite of *Streptomyces,* inhibits protein kinase C (PKC) by interaction with the catalytic domain close to the adenosine triphosphate (ATP)-binding site.[20] PKC is a serine-threonine phosphorylating kinase involved in signal transduction, secretion, desensitization, gene expression, and cell differentiation. The inhibitory effect of staurosporin on PKC is very potent ($K_i \approx 2$ nM),[21] but at higher concentrations other kinases (PKA, calcium-calmodulin-dependent kinase II [CaMKII]) and phosphorylase kinase, are inactivated as well.[22] At 1000 nM of staurosporin, the insulin receptor tyrosine kinase is also inhibited.[23] Staurosporin is known to cause apoptosis in a variety of cell types,[24–26] but the effect on lens epithelial cells has not yet been investigated.

BLECs exposed to staurosporin exhibited a dose-dependent increase in TUNEL-positive cells and in caspase-3 activity. The staurosporin concentration needed for half-maximal response was 240 nM when determining the number of TUNEL-positive cells and 160 nM for caspase-3 activity. The discrepancy between the two dose–response curves may partially be explained by artifacts, because the TUNEL-assay includes extensive washing, leading to loss of apoptotic cells, and thus requiring a higher dose of staurosporin to obtain the same response as for the caspase assay. The dose–response curve for the TUNEL staining also exhibited a second component with a half-maximal response at 15 nM. This component could not be seen for the caspase-3 dose–response curve, suggesting that part of the staurosporin-induced apoptosis is caspase-independent (discussed further later).

Incubation with staurosporin for 24 hours caused apoptosis ranging from 19% to 76% of the cells in different experiments. The apoptotic response for cells from the same culture was very similar. The great variability in apoptotic response between cultures did not seem to be dependent on passage number, nor was there any correlation between proteasome or caspase-3 activity in control cultures and the number of passages (data not shown). The wide range of apoptosis in control and exposed cultures was rather interpreted as phenotypic differences, because BLECs were derived from a number of animals of different ages and gender, and several separate clones were established and used in the experiments. The

TABLE 1. Effect of Lactacystin and/or Staurosporin on BLECs, In Culture and In Vitro, Compared with Caspase-3 and Proteasome Activity and the Number of TUNEL-Positive Cells

Condition	Ratio to Control		
	Number of TUNEL-Positive Cells	Caspase-3 Activity	Proteasome Activity (Chymotrypsin-Like)
Lactacystin (10 μM) in culture versus control without lactacystin	5.57 ± 0.94*	8.72 ± 3.72†	0.14 ± 0.02‡
Staurosporin (1 μM) in culture versus control without staurosporin	149.47 ± 10.90†	14.96 ± 1.52†	0.41 ± 0.11*
Lactacystin (10 μM) and staurosporin (1 μM), both in culture, versus treatment with staurosporin only	0.98 ± 0.11	1.10 ± 0.02	0.39 ± 0.03†
Lactacystin (10 μM) in vitro versus control without lactacystin	—	0.99 ± 0.10	0.15 ± 0.01†
Lactacystin (10 μM) in vitro versus control without lactacystin, both groups after treatment with staurosporin (1 μM) in culture	—	0.79 ± 0.11	0.15 ± 0.01†

All data are expressed as the ratio with corresponding control, as indicated under the condition heading. Statistical evaluation was performed using Student's *t*-test. Data presented are mean ± SD from triplicate culture wells. Representative results from one of three independent experiments.

* $P < 0.01$.
† $P < 0.001$.
‡ $P < 0.05$.

TABLE 2. Caspase-3 Activity and Percentage of TUNEL-Positive Cells after Incubation with 10 μM Lactacystin or 1 μM Staurosporin in the Presence or Absence of the Caspase-3 Inhibitor Z-DEVD-FMK and the Pancaspase Inhibitor Z-VAD-FMK

Group	TUNEL-Positive Cells (%)	Caspase-3 Activity*
Control		
No inhibitor	0.83 ± 0.26	0.192 ± 0.018
Caspase-3 inhibitor	0.55 ± 0.10	0.035 ± 0.008‡
Pancaspase inhibitor	0.55 ± 0.09	0.032 ± 0.003‡
Lactacystin		
No inhibitor	1.67 ± 0.14	1.004 ± 0.105
Caspase-3 inhibitor	2.15 ± 0.35	0.049 ± 0.009‡
Pancaspase inhibitor	2.30 ± 0.34	0.024 ± 0.002‡
Staurosporin		
No inhibitor	30.52 ± 9.28	2.326 ± 0.073
Caspase-3 inhibitor	29.05 ± 3.24	0.078 ± 0.011‡
Pancaspase inhibitor	12.51 ± 0.59†	0.047 ± 0.005‡

P refers to comparison with corresponding control (same group without inhibitor). Data presented are mean ± SD from triplicate culture wells. Representative results from one of three independent experiments.

* Expressed as units of fluorescence per second per gram.

† $P < 0.05$.

‡ $P < 0.001$.

number of apoptotic cells in nontreated cultures was between 0.4% and 1.4%. Whether this reflects the physiological rate of apoptosis in the lens epithelium in situ or is the result of the culturing conditions cannot be elucidated from these experiments, but some rate of cell turnover, which includes apoptosis, is likely to occur in the lens epithelium in vivo.

Staurosporin-induced apoptosis in BLECs caused translocation of PS after approximately 3 hours, and at 6 hours there were signs of apoptotic nuclei (Fig. 2). After 8 hours, some large epithelial cells, which appeared to contain small, condensed apoptotic nuclei were evident, which suggests phagocytosis. A few reports have indicated that lens epithelial cells are capable of phagocytosing apoptotic bodies.[27] Concomitantly with the first appearance of annexin-V-labeled cells, increased activity of caspase-2, -3, -4, -8 ,and -9 was seen (Fig. 4). Other investigators have shown that PS externalization can be prevented by caspase inhibitors,[28,29] thus suggesting translocation of PS as an event downstream of caspase activation. PS-exposure has been demonstrated to precede loss of nuclear lamins, chromatin condensation, and TUNEL-staining.[30–32] This was supported by our data, showing DNA fragmentation after 6 hours of staurosporin treatment.

When describing the function of different caspases, discrimination is generally made between effectors and initiators.[10] The former group, including caspase-2, -3, and -7, is responsible for proteolytic cleavage leading to cell disassembly, whereas the latter (caspase-6, -8, and -9) is involved in upstream regulatory events.[33] A third group comprises caspase -1, -4, and -5. Activation of initiator caspases triggers cleavage of effector caspases, thus leading to amplification of the death signal. Caspase-3 is believed to act on poly-ADP-ribose-polymerase (PARP), a DNA repair enzyme whose expression is triggered by DNA strand breaks. It is believed that cleavage of PARP facilitates the degradation of cellular DNA during apoptosis. Caspase-3 is also known to activate caspase-activated DNase (CAD) by cleavage of a complex between CAD and caspase-activated DNase inhibitor (ICAD), which results in DNA fragmentation. In the present study, there was both a time- and dose-dependent amplification of Ac-DEVD-AMC cleavage by staurosporin, suggesting an important role for caspase-3 in this type of apoptosis. Caspase-3 activity was at least 10-fold higher after 2-hour incubation with staurosporin. This amplification was several times higher than for the other caspases, supporting the general view that caspase-3 is the final link in the caspase-activation cascade and the main effector caspase.

Inhibition of the Proteasome Leads to Apoptosis

The proteasome is a 700-kDa protease complex that is thought to be responsible for turnover of defect proteins during aging, because it prefers oxidatively damaged and ubiquitin-labeled proteins to native proteins.[16,34] In lens, it has been demonstrated that the proteasome prefers mildly photo-oxidized α-crystallins rather than nonoxidized lens proteins.[35] Other proposed roles for the proteasome is in differentiating lens epithelial cells.[36,37] In recent years, the role of proteasome degradation of small short-lived cytoplasmic proteins has gained increasing attention. Such proteins include p53, E2F, c-*myc*, and c-*jun*, known substrates in the ubiquitin-dependent proteasomal degradation pathway and proteins critical for cell cycle progression, transcriptional regulation, and, under certain conditions, involved in regulation of apoptosis.[38] Accumulation of p53, p27, and cyclins D1 and B1 was seen on blocking of the ubiquitin-dependent pathway, leading to caspase-independent apoptosis.[14] However, reports provide contradictory evidence for whether proteasomes are required for, or are protective against, apoptosis.

In this study, inhibition of the proteasome by the highly specific proteasome inhibitor lactacystin caused apoptosis in cultured BLECs. The effect of lactacystin on proteasomal chymotrypsin-like activity was measured by the use of Suc-LLVY-AMC. Incubation of BLEC with 10 μM lactacystin for 25 hours caused 86% reduction of proteasome activity. The in vitro effect of 10 μM lactacystin on Suc-LLVY-AMC hydrolyzing activity was 85%, which is almost the same degree of inhibition as by in culture treatment, indicating good cell permeability of

TABLE 3. Inhibition of Caspase-2, -4, -8, and -9 by Z-VAD-FMK during Incubation with 1 μM Staurosporin for 24 Hours

Caspases	Ratio to Control: Staurosporin, No Inhibitor	Ratio to Control: Staurosporin and Z-VAD-FMK
Caspase-2; Ac-VDVAD-AFC	6.64 ± 0.05	0.59 ± 0.03*
Caspase-3; Ac-DEVD-AMC	12.11 ± 0.38	0.25 ± 0.03*
Caspase-4; Ac-LEVD-AFC	1.49 ± 0.09	0.67 ± 0.04*
Caspase-8; Ac-IETD-AMC	1.41 ± 0.09	0.72 ± 0.09*
Caspase-9; Ac-LEHD-AMC	2.02 ± 0.14	0.91 ± 0.01*

Caspase number and the substrate used are indicated. The pancaspase inhibitor Z-VAD-FMK was used at a concentration of 20 μM. Activity is expressed as the ratio of control activity, i.e., caspase activity in cells not receiving staurosporin treatment. Data are mean ± SD from triplicate culture wells. Representative results from one of three independent experiments.

* $P < 0.001$; comparison with cells incubated with staurosporin but without inhibitor.

this inhibitor. Apoptosis induced by proteasome inhibition caused activation of caspase-3, because there was a concomitant increase in Ac-DEVD-AMC hydrolysis. This increase was not due to a direct effect of lactacystin on caspase-3, because in vitro incubation with lactacystin had no effect on Ac-DEVD-AMC hydrolysis (Table 1).

The mechanism for lactacystin-induced apoptosis is not fully understood. It could be due to unknown side effects of this compound that have no relation to its proteasome inhibiting effect. It has been suggested that blocking proteasome function in a proliferating cell population coincides with arrest in the G_0-G_1 or G_2 phase of the cell cycle, and that this would force the cells to either undergo differentiation or to go into apoptosis,[38] a fate that seems to be dependent on the cell type. Induction of apoptosis in thymocytes by glucocorticoid treatment caused decreased proteasome activities.[39] In this study, chymotrypsin-like activity of the proteasome in BLEC was stable during the first 8 hours of staurosporin-induced apoptosis, although a 59% reduction of activity could be seen after 24 hours (Fig. 5 and Table 1). These data thus suggest that inhibition of the proteasome is not necessary for, but can initiate, apoptosis.

Caspase-Independent Apoptosis

Even though both lactacystin and staurosporin-induced apoptosis leads to activation of caspase-3, inhibition with Z-DEVD-FMK, a caspase-3 inhibitor, or Z-VAD-FMK, a pancaspase inhibitor, did not completely prevent apoptosis, evidenced by TUNEL staining. Preincubation of BLECs with 20 μM Z-VAD-FMK before addition of staurosporin reduced the number of TUNEL-positive cells approximately 60%, but there were still many TUNEL-positive cells, compared with control cultures. Blocking of caspase activation by the pancaspase inhibitor Z-VAD-FMK was very efficient; activity of caspase-2, -3, -4, -8, and -9 was lowered beneath the endogenous level of activity (i.e., the activity in control cells, excluding the possibility of incomplete inhibition). Recent experiments have shown that staurosporin-induced apoptosis in activated peripheral T lymphocytes can occur in the presence of broad-spectrum peptide caspase inhibitors[40] and therefore independently of caspases. Cytoplasmic features of apoptosis such as cell shrinkage, PS externalization, and loss of mitochondrial membrane potential were seen, but neither degradation of nuclear substrates such as PARP and lamins occurred, nor did DNA fragmentation or extreme chromatin condensation. In this study, PS externalization occurred during staurosporin incubation despite caspase inhibition, thus supporting the current findings. However, as shown in Figure 6F (inset), caspase-independent nuclear fragmentation could also be seen. The present study thus demonstrates complete apoptosis of BLECs exposed to staurosporin in the presence of a pancaspase inhibitor, which is in contrast to previous reports.

Incubation of BLECs with staurosporin of increasing concentrations resulted in dose–response curves for TUNEL positivity and caspase-3 activity with slightly different requirements of concentration for half-maximal response. This may be due to artifacts such as those caused by extensive washing during the TUNEL-assay, but may also indicate that caspase activity and DNA-fragmentation are not as tightly coupled, as was previously assumed. The finding of an extra component in the dose–response curve of TUNEL-positive cells indicates that

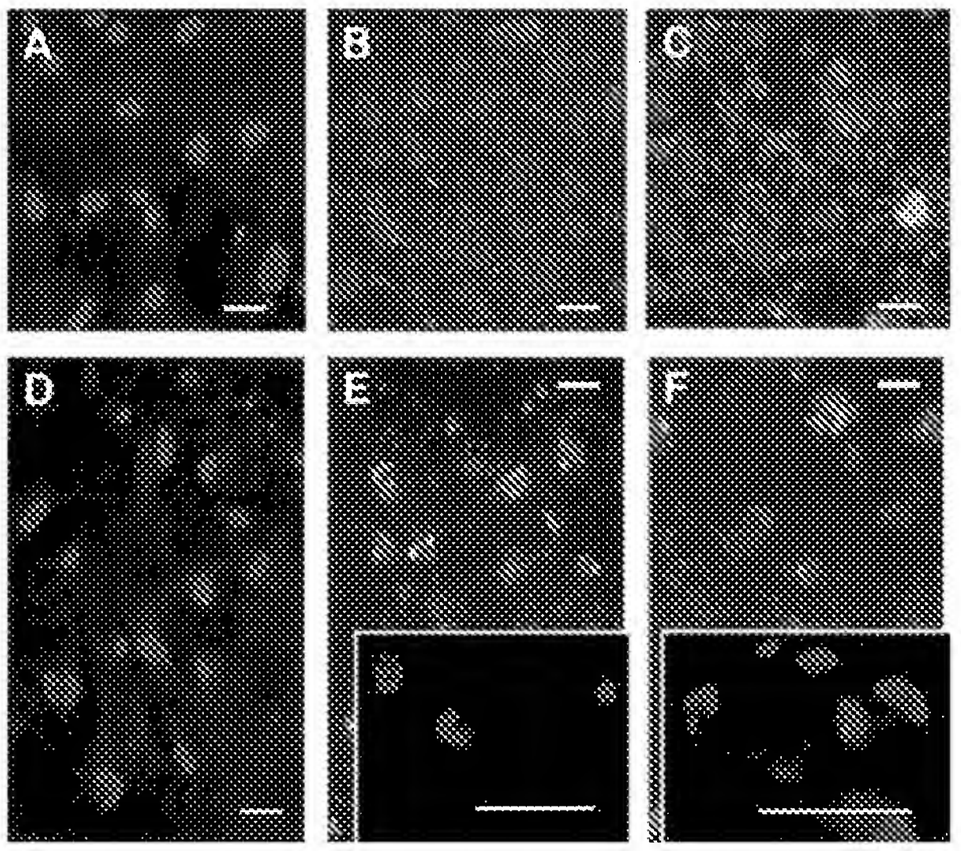

FIGURE 6. BLECs stained with Hoechst 33342 (*blue*), annexin V (*green*), and propidium iodide (*red*). Control cells (**A**, **D**) and cells incubated with 1 μM staurosporin in the absence (**B**, **E**) or presence (**C**, **F**) of the pancaspase inhibitor Z-VAD-FMK. Cells were stained after 3 (**A**, **B**, and **C**) or 6 (**D**, **E**, and **F**) hours. Annexin-V labeling occurred after 3 hours of staurosporin incubation both in cells not treated with Z-VAD-FMK (**B**) and in those preincubated with caspase inhibitor (**C**). After 6 hours of staurosporin treatment, cells showed nuclear fragmentation (**E**, **F** insets; Hoechst stain). This was apparent both with (**E**, inset) or without (**F**, inset) inhibitor. Scale bar, 50 μm.

there may be levels or intervals of apoptotic stimuli intensity (in this case range of staurosporin concentration) where apoptosis is totally independent of caspase activation. This study is the first to demonstrate that lens epithelial cells have a caspase-independent apoptotic pathway that can be activated by staurosporin or by inhibition of the proteasome. Although these data support recent findings in other cell types that apoptosis can be caspase independent, further studies are needed to elucidate alternative mechanisms. Blocking of the ubiquitin-dependent pathway has been reported to be caspase independent,[14] which is confirmed by the present data because caspase inhibition did not prevent lactacystin-induced apoptosis. In hematopoietic progenitor cells, DNA fragmentation caused by lactacystin could be prevented by the protease inhibitor N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), which inhibits chymotrypsin-like and some cysteine proteases.[41] The identity of the protease or proteases responsible for the final steps in caspase-independent apoptosis is still unknown, and there is the possibility of additional caspases that have not yet been discovered.

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